

# Phage–bacteria network analysis and its implication for the understanding of coral disease

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## Summary

Multiple studies have explored microbial shifts in diseased or stressed corals; however, little is known about bacteriophage interactions with microbes in this context. This study characterized microbial 16S rRNA amplicons and phage metagenomes associated with *Montastraea annularis* corals during a concurrent white plague disease outbreak and bleaching event. Phage consortia differed between bleached and diseased tissues. Phages in the family *Inoviridae* were elevated in diseased or healthy tissues compared with bleached portions of diseased tissues. Microbial communities also differed between diseased and bleached corals. Bacteria in the orders *Rhodobacterales* and *Campylobacterales* were increased while *Kiloniellales* was decreased in diseased compared with other tissues. A network of phage–bacteria interactions was constructed of all phage strains and 11 bacterial genera that differed across health states. Phage–bacteria interactions varied in specificity: phages interacted with one to eight bacterial hosts while bacteria interacted with up to 59 phages. Six phages were identified that interacted exclusively with *Rhodobacterales* and *Campylobacterales*. These results suggest that phages have a role in controlling stress-associated bacteria, and that networks can be utilized to select potential phages for mitigating detrimental bacterial growth in phage therapy applications.

## Introduction

Coral reefs are considered some of the most diverse environments in the world (Moberg and Folke, 1999;

Veron *et al.*, 2009). Scleractinian (stony) corals generate the physical structure of coral reefs, and thus are ecologically important members of many tropical marine ecosystems. In addition to supporting macroscopic reef communities, corals also host diverse microbial consortia that include *Symbiodinium* dinoflagellates, bacteria, archaea, fungi and other microbial eukaryotes (Knowlton and Rohwer, 2003; Cróquer *et al.*, 2006). Culture-based studies have demonstrated that these coral-associated microbes (the coral microbiota) take on a variety of roles ranging from mutualistic to pathogenic (Rohwer *et al.*, 2001; Ritchie, 2006; Mouchka *et al.*, 2010; Rosenberg and Kushmaro, 2011; Cook *et al.*, 2013). For example, while bacteria such as *Serratia marcescens*, *Vibrio shiloi* and *Vibrio coralliilyticus* have been shown to cause disease signs (Ben-Haim, 2003; Rosenberg and Falkovitz, 2004; Sutherland *et al.*, 2011), *Photobacterium* spp. cultured from coral mucus secrete antibiotics that can reduce pathogen growth (Ritchie, 2006).

The coral microbiota has been posited to play important roles in mediating the effects of global environmental changes on coral health (Williams *et al.*, 1987; Rosenberg *et al.*, 2007; Ainsworth *et al.*, 2010). The health of coral reefs is an issue of concern due to changing environmental conditions, including the eutrophication of water due to coastal development, overfishing of herbivores, ocean acidification and increased sea surface temperatures (Harvell *et al.*, 1999; Nyström *et al.*, 2000; Pandolfi *et al.*, 2005; 2011; Vega Thurber *et al.*, 2014). These environmental stressors leave the corals vulnerable to disruption of their microbial symbioses, including susceptibility to opportunistic pathogenesis. For example, temperature stress can lead to bleaching, a phenomenon where the symbiotic dinoflagellates (*Symbiodinium* spp.) are either expelled or degraded, leaving the coral host bereft of its main energy source (Brown, 1997; McClanahan *et al.*, 2009; Tolleter *et al.*, 2013). Interactions in the coral microbiota may inhibit or exacerbate this bleaching. For example, *Vibrio* spp. have been shown to cause bleaching in corals that are heat stressed (Kushmaro *et al.*, 2001; Ben-Haim and Rosenberg, 2002; reviewed in Rosenberg *et al.*, 2007). Therefore, stress can induce changes in the microbiota that lead to additional stress or disease (Brandt and McManus, 2009).

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Although there are over three dozen coral diseases described, most have no known aetiological agent (Green and Bruckner, 2000; Sutherland *et al.*, 2004; Rosenberg *et al.*, 2007; Bourne *et al.*, 2009; Pollock *et al.*, 2011). Some of these diseases may represent generic responses to stress (Kuntz *et al.*, 2005) that ultimately lead to opportunistic infection by diverse combinations of copiotrophic bacteria. These opportunistic bacteria are typically ubiquitous in the environment but replicate in corals under conditions where the host is stressed or immune compromised, which may be the result of another infection (Burge *et al.*, 2013). Some examples of these hypothesized opportunistic bacteria include members of orders *Rhodobacterales*, *Campylobacterales*, *Clostridiales* and *Vibrionales* (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Rosenberg *et al.*, 2007; Sekar *et al.*, 2008; Sunagawa *et al.*, 2009; Vega Thurber *et al.*, 2009; Mouchka *et al.*, 2010). Viruses also infect many members of the coral holobiont including the coral animal itself (Vega Thurber *et al.*, 2008), the photosynthetic *Symbiodinium* algae (Correa *et al.*, 2012) and bacteria associated with coral mucus, tissue or skeleton (Wegley *et al.*, 2007; Marhaver *et al.*, 2008; Vega Thurber *et al.*, 2008; Littman *et al.*, 2011). Although the diversity and roles of eukaryotic viruses associated with coral diseases and bleaching have been recently described (e.g. Soffer *et al.*, 2014; Littman *et al.*, 2011), less is known about the roles of bacteriophages. Bacteriophages can influence their bacterial hosts positively and negatively (Rohwer and Vega Thurber, 2009). For example, lytic phages are estimated to cause  $10^{28}$  infections per day (Suttle, 2007). Yet some lysogenic phages prevent infection from other phages and/or add new beneficial genes via horizontal gene transfer, potentially increasing bacteria host fitness as a result (Mann *et al.*, 2003; Brüßow *et al.*, 2004; Lindell *et al.*, 2007; Breitbart, 2012).

Previous metagenomics studies have determined that myo-, siphon-, micro- and podophages are found in coral mucus/tissue (Wegley *et al.*, 2007; Marhaver *et al.*, 2008; Vega Thurber *et al.*, 2008; Littman *et al.*, 2011). It is likely that interactions among bacteriophage and the coral bacteria and archaea result in alterations of the microbiome such as controlling coral infection from either bona fide pathogens or opportunists. For example, the application of host-specific phages to diseased individuals has been shown to prevent *Vibrio* disease in Red Sea *Favia* spp. corals. This form of 'phage therapy' has been suggested to be used for potential control of disease-causing bacteria in natural settings (Efrony *et al.*, 2007; 2009; Atad *et al.*, 2012). However, unless the target host is amenable to culturing, identifying the phages that associate with target bacteria and can therefore be used for phage therapy will be constrained. Since 35–90% of bacteria from the environment are not

cultivable, we are limited in our understanding of the interactions between the coral microbiota and its phage predators (Rappé and Giovannoni, 2003; Cook *et al.*, 2013). Furthermore, it has been difficult to predict bacteria–phage interactions as phages have variable host ranges and bacteria differ in their susceptibility to individual phages or multiple phages (Flores *et al.*, 2011; Weitz and Wilhelm, 2012). Yet these complex and underexplored top-down forces likely influence the overall microbial ecology of corals and coral reefs (Rohwer and Thurber, 2009).

Networks of interactions between bacteria and bacteria, bacteria and eukaryotes, phage-encoded bacterial genes, or proteins and their metabolites (metabolic networks) are increasingly used to describe complex ecosystem interactions, and similar methods can be applied to determine interactions among uncultured phage and bacteria from host samples (Zhou *et al.*, 2010; Steele *et al.*, 2011; Faust and Raes, 2012; Faust *et al.*, 2012; Faust and Raes, 2012; Rodriguez-Lanetty *et al.*, 2013; Modi *et al.*, 2013; Chow *et al.*, 2013).

Here we investigate the communities of bacteria, archaea and bacteriophages associated with *Montastraea annularis* (also referred to as *Orbicella annularis*) corals during a simultaneous outbreak of white plague (WP) disease and coral bleaching in the US Virgin Islands. By comparing microbial and bacteriophage estimated relative abundances across conditions, we sought to gain new insights into the ecological interactions between phage and bacteria. Using a combination of 16S rRNA gene amplicon and shotgun metagenomic analyses, we found that bacterial and phage communities varied among different health states, with bacterial types previously determined to be affiliated with coral disease and other stress conditions (e.g. *Campylobacterales* and *Rhodobacterales*) elevated in diseased corals (Cooney *et al.*, 2002; Frias-Lopez *et al.*, 2002; Rosenberg *et al.*, 2007; Sekar *et al.*, 2008; Sunagawa *et al.*, 2009; Vega Thurber *et al.*, 2009; Mouchka *et al.*, 2010). We also constructed a network using multiple correlation methods with CoNet (Faust and Raes, 2012; Faust *et al.*, 2012) in Cytoscape (Shannon *et al.*, 2003). We identified six phage strains that interacted exclusively with two bacteria enriched in WP disease, but did not interact with any bacteria that were enriched in healthy corals (and therefore may be mutualists). These exclusive interactions suggest that such phages could be potential candidates on which to focus culturing efforts for phage therapy against opportunistic bacterial infections arising during WP outbreaks. This network analysis allowed us to infer phage–bacterial interactions from our non-cultured environmental coral samples and highlight their potential roles in coral microbial ecology and phage therapy of coral diseases.

## Results and discussion

### *Corals were dominated by Proteobacteria and Bacteroidetes*

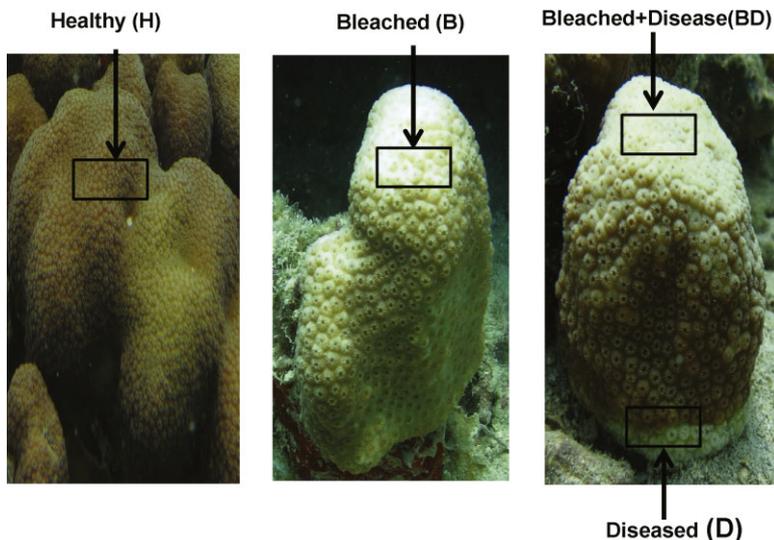
16S rRNA microbial libraries were constructed from coral tissues sampled during a concurrent WP disease outbreak and bleaching event in the US Virgin Islands in 2010. Coral samples were taken from four health states: healthy (H), bleached (B), bleached + diseased (BD) and WP diseased (D) (Fig. 1). The BD tissues were sampled from bleached portions of the diseased colony not showing signs of WP, while D tissues were sampled at the leading edge of the disease lesion. In total, 68 577 amplicon sequences passed quality filtering across the 22 samples. 16S rRNA gene amplicon sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity, and the representative sequence for each OTU was assigned taxonomy using the Ribosomal Database Project (RDP) classifier at a 70% confidence threshold. Bacterial and archaeal OTUs were assigned to 417 genera within 175 bacterial or archaeal orders and 38 phyla (Table S1). One thousand three hundred and two sequences, or 1.9% total, could not be assigned to any domain. Sequencing depth ranged from 957 to 5458 (mean 3117). Seven of these 175 orders (4.0%) were Archaea including similarities to the *Cenarchaeales* within the phylum *Crenarchaeota* and three members of the *Euryarchaeota* (E2, WCHD3-30 YLA114). These archaea are within the same phyla that have previously been found in corals using archaeal-specific primers (Kellogg, 2004; Wegley *et al.*, 2004; Beman *et al.*, 2007), but most of the archaea identified here were both low abundance and low prevalence: none of the seven archaeal relative mean abundances rose above 0.35% per sample, and Archaea were entirely absent in 42% of the libraries. Archaea thus

appear to be a numerically minor constituent of the tissue-associated community, similar to previous studies examining combined bacterial and archaeal communities in corals (Vega Thurber *et al.*, 2009; Sato *et al.*, 2013). The remaining 167 orders for which taxonomy was assigned were bacterial.

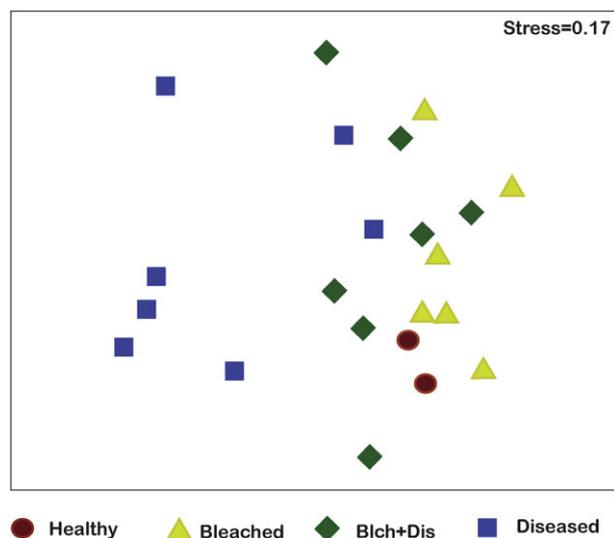
Comparison of the abundance of phyla did not reveal differences across health states (Kruskal–Wallis,  $P > 0.05$ ). The phylum *Proteobacteria* was most common in all the libraries [ $57 \pm 5\%$  mean relative abundance  $\pm$  standard error of the mean (SEM)], followed by *Bacteroidetes* ( $23 \pm 2\%$  mean relative abundance  $\pm$  SEM). *Proteobacteria* abundances did not significantly differ across health states (Kruskal–Wallis  $P > 0.05$ ), corroborating previous studies on both WP and healthy 16S libraries (Pantos *et al.*, 2003; Cárdenas *et al.*, 2012; Kellogg *et al.*, 2012). *Bacteroidetes* were also not significantly different across health states (Kruskal–Wallis  $P > 0.05$ ), but this finding is in contrast with past studies that showed elevations of this taxa in WP and other diseased or stressed corals (Pantos *et al.*, 2003; Barneah *et al.*, 2007; Vega Thurber *et al.*, 2009; Garcia *et al.*, 2013).

### *Bleached and WP diseased tissues harbour distinct microbes*

To assess whether there were microbial shifts in WP, as well as bleached and healthy corals, whole bacterial and archaeal communities at the genus level were compared using Bray–Curtis distances, and tested for significance with multivariate statistics. Microbial communities from WP disease (D) were significantly different from both bleached regions of corals (BD and B) [analysis of similarity (ANOSIM)  $P \leq 0.05$ ; Global  $R = 0.29$ ], yet no



**Fig. 1.** Coral health states sampled for bacteriophage and microbe metagenomes. Black boxes indicate sampling scheme. H samples were taken from corals showing no signs of either bleaching or white plague. B samples were taken from the bleached portion of bleached coral colonies with no visible signs of white plague. BD samples are taken from corals affected by both bleaching and white plague disease, and were taken from bleached tissues not in contact with the leading edge of white plague tissue loss. D tissues were sampled from the leading edge of white plague tissue loss, and included only live tissue with no signs of paling or bleaching.



**Fig. 2.** Diseased and bleached microbial consortia are different. Non-metric multidimensional scaling (MDS) plot of microbial communities from different health states at the genus taxonomic level. A log-transformed Bray–Curtis resemblance matrix was used to construct the 2D MDS. Microbial communities from D tissues significantly differed from communities in B and BD tissues (ANOSIM  $P \leq 0.05$ ; Global R = 0.29). No tissue type clustered significantly separately from H (ANOSIM  $P \leq 0.05$ ; Global R = 0.29). Stress value is based on 2D MDS. Symbols are: healthy (red circles); bleached (yellow triangles); bleached and diseased (green diamonds); diseased (blue squares).

communities were significantly different from healthy corals (ANOSIM  $P > 0.05$ ; Global R = 0.29) (Fig. 2). However, due to the high prevalence of disease and bleaching in the sampled area, there were only two healthy samples available for this study. Nevertheless, there are clear differences in the bleached and diseased tissue's overall microbial communities. One interpretation of this shift is that loss of symbionts (bleaching) contributes to alteration of the microbial community (*sensu* Littman *et al.*, 2010) which is one of the major distinctions between tissue composition of D and BD, other than proximity to the disease lesion.

#### *Campylobacteriales* and *Rhodobacteriales* are elevated in diseased tissues

To assess which bacteria are associated with WP tissues, we first searched for the original proposed WP pathogen, *Aurantimonas corallicida* (an  $\alpha$ -*Proteobacterium* in the order *Rhizobiales*) (Richardson *et al.*, 1998). *Aurantimonas* sequences were only found in one bleached sample (~0.5% relative abundance) but not in any WP-affected corals (D or BD) in this study. Instead, the orders *Campylobacteriales* and *Rhodobacteriales* were elevated in D tissues compared with all others (Table 1). These orders are associated with corals afflicted with

a variety of other diseases and general stressors (Frias-Lopez *et al.*, 2002; Cooney *et al.*, 2002; Sekar *et al.*, 2008; Sunagawa *et al.*, 2009; Mouchka *et al.*, 2010). When comparing microbial genera rather than orders, a similar pattern was found such that significant increases were seen in the relative abundance of the genus *Arcobacter* (order *Campylobacteriales*) and an unknown genus in the order *Rhodobacteriales* in D tissues ( $P \leq 0.05$ ) (Fig. 3). *Vibrios*, which are typically associated with various diseased and stressed corals (Kushmaro *et al.*, 2001; Ben-Haim and Rosenberg, 2002; Cervino *et al.*, 2004), did not vary significantly across health states, indicating that this genus is unlikely to have a role in bleaching or WP disease in this case. Additionally, *Vibrios* were found in low mean abundances (< 0.6%) in all health states.

Although members of the order *Rhodobacteriales* are known associates of healthy corals, they are often found at higher relative abundance in various diseased corals, suggesting that it is an opportunistic bacterial group that proliferates during environmental perturbations (Mouchka *et al.*, 2010). For example, *Rhodobacteriales* has been previously found to be more abundant in WP infected *Montastraea faveolata* than healthy specimens (Sunagawa *et al.*, 2009). In addition, *Rhodobacteriales* have been found to be enriched in black band disease affected *M. annularis* (Cooney *et al.*, 2002) and *Sidestreaea* spp. (Sekar *et al.*, 2008). Sharp and colleagues (2012) showed that *Rhodobacteriales* are associated with corals in early life stages, as soon as 4 days post-fertilization, which could indicate that *Rhodobacteriales* are bacteria that thrive in the early succession of microorganisms during coral development. In other contexts, stress or disease have been shown to create disturbances that allow early successional ('weedy') microorganisms to flourish (Koenig *et al.*, 2011; Lozupone *et al.*, 2012; Shade *et al.*, 2012). Likewise, stress or disease (e.g. WP) can also create disturbed microbial communities similar to a transitional/fluctuating stage that may lead to *Rhodobacteriales* to opportunistically infect the coral.

Tissue samples adjacent to WP lesions (D), but not bleached regions at the top of the same colonies (BD),

**Table 1.** Significantly different bacterial orders.

Bacteria order	Significant pairwise differences	Kruskal–Wallis statistic	<i>P</i> -value
<i>Campylobacteriales</i>	H-D; B-D; BD-D	18.57	0.0001
<i>Kiloniellales</i>	H-D; B-D; BD-D	8.57	0.04
<i>Rhodobacteriales</i>	H-D; BD-D	10.45	0.02

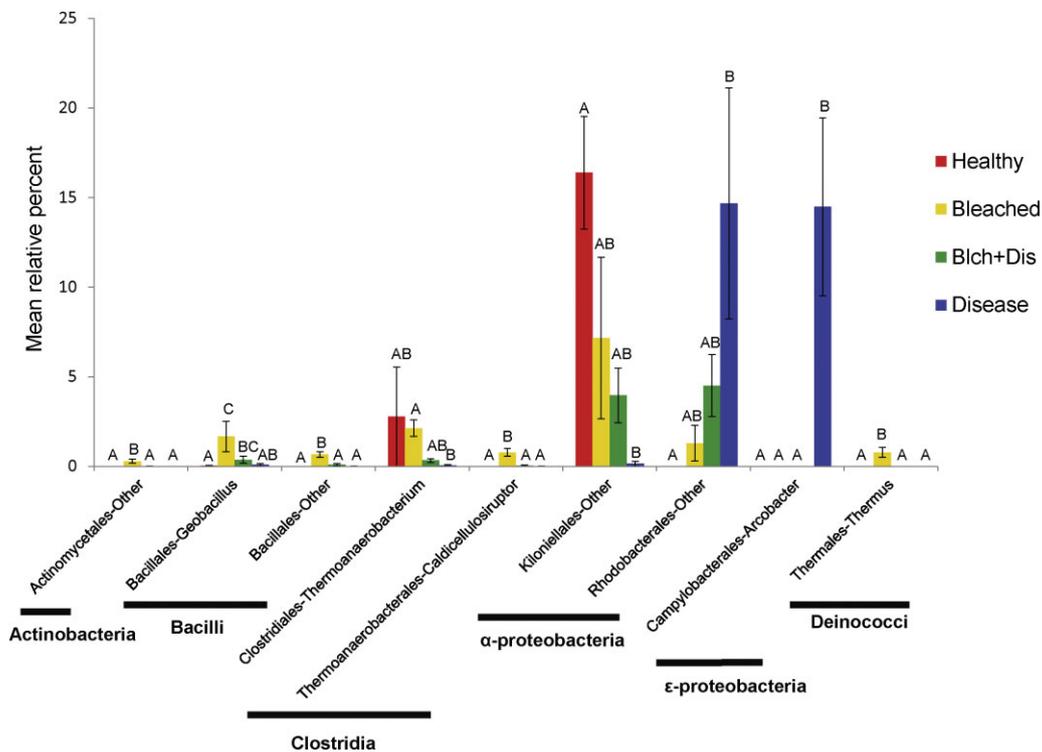
Significance based on Kruskal–Wallis and Bonferroni *post hoc* pairwise tests.

were enriched in *Arcobacter*, an epsilon proteobacterium in the order *Campylobacteriales*. While some *Campylobacteriales* are non-pathogenic nitrogen fixers (McClung *et al.*, 1983), the genus *Arcobacter* includes known pathogens of humans and other animals (Ho *et al.*, 2006; Collado and Figueras, 2011). In *M. annularis*, *Campylobacteriales* (including sequences identified as *Arcobacter*) have previously been observed at higher mean abundance in Black band disease mats than surrounding seawater, healthy tissue or exposed skeleton (Frias-Lopez *et al.*, 2002). Furthermore, Frias-Lopez and colleagues (2002) found that *Arcobacter* was increased in exposed skeleton adjacent to Black band disease mats in *M. cavernosa*. It therefore seems likely that the *Arcobacter* found enriched at the boundary of WP lesions in our study either contribute to, or opportunistically benefit from, WP disease. However, further study is needed to distinguish these possibilities. For example, cultured *Arcobacter* could be added to healthy individuals and if signs of the WP are recapitulated then it likely is involved in the disease. Alternatively, *Arcobacter* would be added to WP-infected corals in aquaria to test whether *Arcobacter* sp. have a measurable effect on the health of

corals suffering from WP disease, or if these bacteria passively benefit from nutrients and/or niche space liberated by the receding coral tissue.

#### *Kiloniellales are lost in diseased tissues*

Another shift in bacterial communities detected in WP was the loss of an unknown genus in the order *Kiloniellales*, which is a newly described bacterial order in the  $\alpha$ -*Proteobacteria* (to which the *Rhodobacteriales* also belong). *Kiloniellales* were previously isolated from macroalgae and the surface mucus layer of coral larvae (Wiese *et al.*, 2009; Sharp *et al.*, 2012; Cleary *et al.*, 2013). Interestingly, this was the only genus found to be significantly more abundant in healthy tissues compared with D (Kruskal–Wallis  $P \leq 0.05$ ) (Table 1, Fig. 3). This may indicate that these microorganisms are members of the healthy tissue community that are lost during disease, although their contribution to host fitness (if any) is currently unknown. Future studies on the presence of *Kiloniellales* in corals and a description of its metabolic functions would help further determine the role of this bacterial group in coral health.



**Fig. 3.** Multiple bacterial genera are significantly different across health states. Relative abundance for bacterial genera found to significantly vary across health states (Kruskal–Wallis,  $P \leq 0.05$ ) is plotted on the y-axis. The x-axis labels each order and genus (separated by a dash). Horizontal bars and bold labels group genera in the same phylum. Bar colours represent the health states: healthy (red); bleached (yellow); bleached + diseased (green); diseased (blue). Letters indicate significance groups: bars that share at least one letter were not significantly different from one another and bars without shared letters were significantly different (Kruskal–Wallis,  $P \leq 0.05$ ). Error bars represent standard error of the mean. Relative abundance values for genera that differed across health states are reported as a portion of the whole microbial community, and therefore do not add up to 100%.

### *Bleached corals harbour low abundance taxa similar to known thermally tolerant bacteria*

Multiple bacterial genera were significantly more abundant in B corals than all of the other health states (Kruskal–Wallis,  $P \leq 0.05$ ), including two thermally tolerant genera: *Caldicellulosiruptor* (order *Thermoanaerobacterales*) and *Thermus* (order *Thermales*) (Fig. 3). Another taxon similar to a thermally tolerant genus, *Thermoanaerobacterium* (order *Clostridiales*), was found to be more abundant ( $P \leq 0.05$ ) in B tissues relative to D tissues (Fig. 3). Additionally, a genus from each order *Actinomycetales* and *Bacillales* was found in higher relative abundance in bleached corals than the other health states. However, some of these bacteria were not in higher relative abundance in the BD tissues, so it is possible that other factors are involved than bleaching alone. Although these bacterial types were found to be significantly more relative abundant ( $P \leq 0.05$ ), they are still at low percentages ( $< 2\%$ ) of the microbiome, and their effects (if any) on coral bleaching remains to be determined.

### *Bacteriophage consortia differ between disease and bleached corals*

To evaluate the differences in coral phage consortia among health states, we generated viral shotgun metagenomic libraries using standard methods (Vega Thurber *et al.*, 2009), and annotated them using tBLASTx (E-val  $\leq 10^{-5}$ ) to the CAMERA viral database (Sun *et al.*, 2011). Of 944 509 total metagenomic reads, there were a total of 70 927 distinct high-quality sequences ( $\sim 500$  bp) similar to viruses, of which 52 598 (74%) were similar to bacteriophages. ANOSIM analysis of Bray–Curtis distances between consortia of phage strains (as measured by the estimated relative abundances in each sample) detected differences among the health states. The results were similar to the pattern of bacterial communities: D consortia clustered separately from both bleached samples (B and BD) ( $P \leq 0.05$ : Global R = 0.233), but none were significantly different from healthy corals ( $P > 0.05$ : Global R = 0.233) (Fig. S1). The phage consortia consisted of 519 total strains, falling into five phage families: *Inoviridae*, *Myoviridae*, *Microviridae*, *Podoviridae* and *Siphoviridae*. *Myo*-, *Podo*- and *Siphoviridae* are dsDNA phages in order *Caudovirales*, while *Inoviridae* and *Microviridae* are ssDNA phages not currently assigned an order. Furthermore, to understand the roles that phage may play in WP and/or bleaching, we tested whether phage families exhibited general differences across health states. BD tissues had significantly lower *Inoviridae* abundances than H or D tissues (Kruskal–Wallis,  $P \leq 0.05$ ). Unclassified phages were decreased in H compared with B, but not compared with BD or D

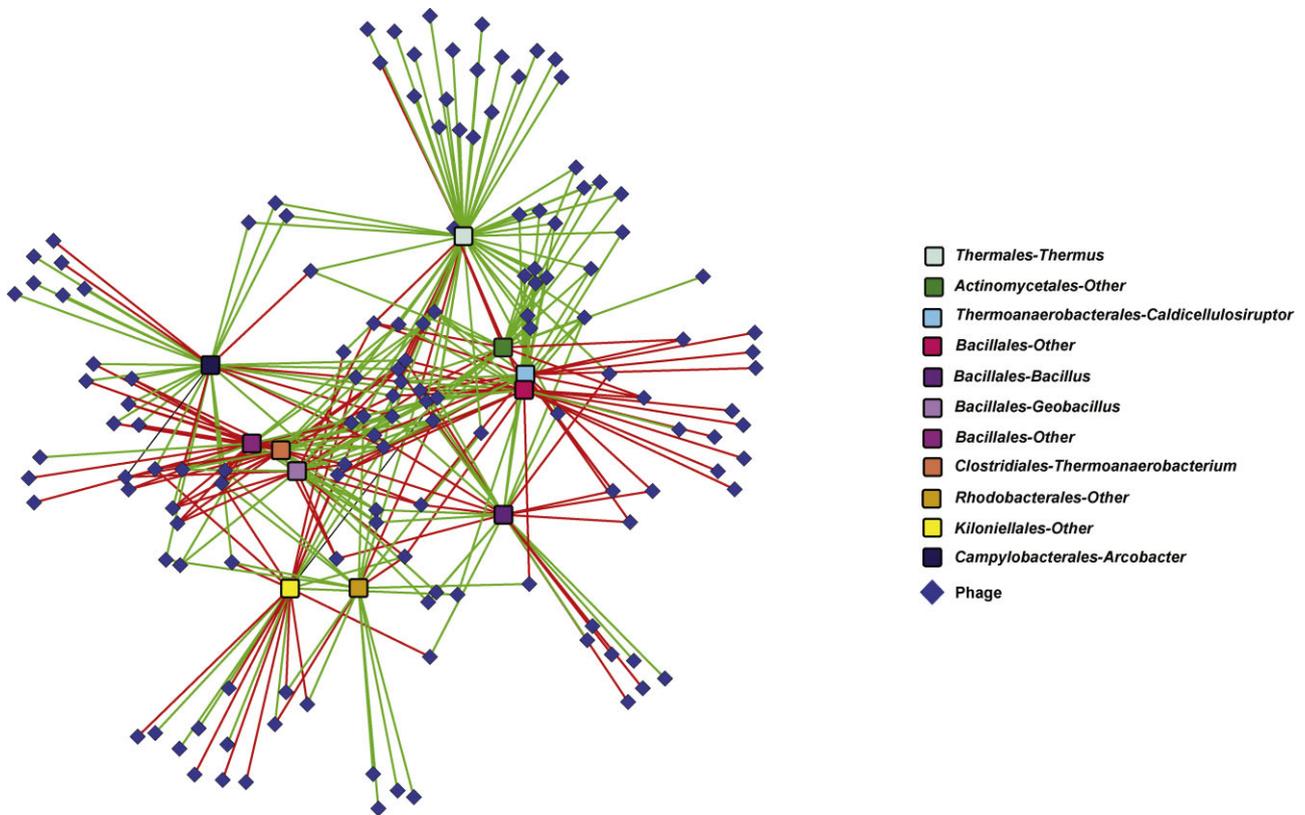
(Kruskal–Wallis,  $P \leq 0.05$ ). However, phage in families *Microviridae*, *Myoviridae*, *Podoviridae* and *Siphoviridae* did not vary across health states (Kruskal–Wallis,  $P > 0.05$ ) (Fig. S2).

### *Phage–bacteria networks reveal hubs of interaction*

In order to predict interactions (either co-occurrence or mutual exclusion) among bacteria and phages, we constructed a network of correlations between the abundance of bacterial genera and phage strains from all health states (Fig. 4). Similar methods have been successfully applied to study bacteria–bacteria (Faust and Raes, 2012; Rodriguez-Lanetty *et al.*, 2013), bacteria–eukaryote (Steele *et al.*, 2011) and metabolic networks in sequence data (Zhou *et al.*, 2010; reviewed in Faust and Raes, 2012; Faust *et al.*, 2012). Because we were specifically interested in phage control of disease-associated bacterial types, only the 11 bacterial genera that differed among health states [analysis of variance (ANOVA) and false discovery rate (FDR) ( $q = 0.2$ ) in QIIME] were compared against all 519 detected phage strains.

In the resulting network of significant phage–bacteria interactions, each node is either a bacterial genus (Fig. 4, square symbols) or phage strain (diamond symbols), and edges indicate significant co-occurrence (green) or mutual exclusion (red) interactions. The network included 151 nodes and 332 edges (representing 205 co-occurrence and 125 mutual exclusion interactions). Co-occurrence relationships between phage and bacterial abundances reflect several likely interactions: (i) the increase of bacterial host abundance directly allows growth of phage populations, (ii) the increased phage populations remove a major competitor of the co-occurring bacterial population, and (iii) the increased phage populations directly infect and benefit the co-occurring bacterium (e.g. by facilitating gene transfer). By contrast, mutual exclusion relationships might reflect: (i) lytic phages directly reducing bacterial populations or (ii) bacteria competing with the excluded phage's host, lowering phage populations.

In addition to identifying interactions between phage–bacteria pairs, some network analysis allows descriptions of the properties of overall interaction structure. The node degree distribution of the resulting phage–bacteria network approximately fit a power law ( $R^2 = 0.83$ ) and therefore had properties of a scale-free network (i.e. non-random network) (Barabási, 1999) (Fig. S3). True scale-free networks are characterized by short paths separating nodes. Biologically, these short average path lengths have been interpreted as causing scale-free networks to respond rapidly to perturbation (Faust and Raes, 2012). We calculated the Average Shortest Path Lengths (AL) for the network and compared them against other biological interaction networks. The resulting AL for this network was



**Fig. 4.** Predicted phage–bacteria interaction network. The network was calculated for the 11 bacterial genera contributing to health state community dissimilarity. Squares: bacteria; diamonds: phage. Bacterial labels are order and genus separated by a dash. Red lines connecting phage–bacteria pairs indicate significant mutual exclusion patterns, while green lines denote significant co-occurrence patterns ( $P \leq 0.05$ ) as determined by the ReBoot randomization procedure in the CoNet Cytoscape package (Faust and Raes, 2012; Faust *et al.*, 2012). Network layout was calculated using edge-weighted spring embedded layout in Cytoscape (Shannon *et al.*, 2003).

3.10, indicating that roughly three edges separate average nodes. This value was similar to those in networks of marine microbial (bacteria and archaea: 3.05; bacteria, protist and virus: 3.00) interactions, which are considered to have small AL (Steele *et al.*, 2011; Faust and Raes, 2012; Chow *et al.*, 2013).

The scale-free properties of this network suggests that bacterial types that are prone to phage interactions (such as *Thermus* that had a total 59 phage interactions; Table 2) are likely to be targeted by new phages added to the network, or that it is a generalist host. Along those lines, the heterogeneity value in networks describes the possibility of hubs. The heterogeneity value of this network was 1.799, almost double the value determined for a network of T4 virus–bacteria interactions from surface seawater (0.953) (Chow *et al.*, 2013). Hubs are thought to describe keystone species that, if removed, would lead to large changes in the community (Steele *et al.*, 2011). Thus, coral tissue networking may have more ‘keystone’ microbes and phages than seawater. A possible keystone might be the phage similar to *Geobacillus*-phage-GBSV1. This phage was predicted to

interact with eight bacterial genera, the maximal number of interactions (all co-occurrence) that was determined for any single phage (Table S2, Fig. S3) in this network. It is plausible that targeted removal of this phage would thus lead to greater changes in the bacterial community than the removal of the phage similar to *Vibrio*-phage-KV40, for example, which only interacts with one bacterial genus (Table S2, Fig. S4). Conversely, addition of this phage may alter communities where it is absent.

#### *Theoretical use of networks to predict candidates for phage therapy*

Phage therapy has been shown to be effective against *Vibrio* infections of corals (Efrony *et al.*, 2007; 2009; Atad *et al.*, 2012). Although it may be impractical to add phages to reefs to prevent or mitigate infections, these therapies may be useful in controlled coral culture settings where high densities may lead to disease (Sheridan *et al.*, 2013). In other contexts such as fish (Nakai and Park, 2002) and shrimp hatcheries (Karunasagar *et al.*, 2007), phage therapy is being explored as a method for pathogen

**Table 2.** Number of phages interacting with each bacteria.

Bacteria phylum; class; order; family; genus	Co-occurrence	Mutual exclusion	Total phage interactions	Single partner interactions
<i>Actinobacteria</i> ; c – <i>Actinobacteria</i> ; o – <i>Actinomycetales</i> ; f – <i>Promicromonosporaceae</i> ; Other	20	6	26	1
<i>Firmicutes</i> ; c – <i>Bacilli</i> ; o – <i>Bacillales</i> ; f – <i>Bacillaceae</i> ; g – <i>Bacillus</i>	17	13	30	11
<i>Firmicutes</i> ; c – <i>Bacilli</i> ; o – <i>Bacillales</i> ; f – <i>Bacillaceae</i> ; g – <i>Geobacillus</i>	11	11	22	0
<i>Firmicutes</i> ; c – <i>Bacilli</i> ; o – <i>Bacillales</i> ; f – <i>Bacillaceae</i> ; Other	12	16	28	7
<i>Firmicutes</i> ; c – <i>Bacilli</i> ; o – <i>Bacillales</i> ; f – <i>Sporolactobacillaceae</i> ; Other	19	16	35	14
<i>Firmicutes</i> ; c – <i>Clostridia</i> ; o – <i>Clostridiales</i> ; f – <i>Clostridiaceae</i> ; g – <i>Thermoanaerobacterium</i>	10	18	28	1
<i>Firmicutes</i> ; c – <i>Clostridia</i> ; o – <i>Thermoanaerobacteriales</i> ; f – <i>Caldicellulosiruptoraceae</i> ; g – <i>Caldicellulosiruptor</i>	14	13	27	6
<i>Proteobacteria</i> ; c – <i>Alphaproteobacteria</i> ; o – <i>Kiloniellales</i> ; f – ; g –	9	15	24	19
<i>Proteobacteria</i> ; c – <i>Alphaproteobacteria</i> ; o – <i>Rhodobacterales</i> ; f – <i>Rhodobacteraceae</i> ; Other	15	3	18	6
<i>Proteobacteria</i> ; c – <i>Epsilonproteobacteria</i> ; o – <i>Campylobacteriales</i> ; f – <i>Campylobacteraceae</i> ; g – <i>Arcobacter</i>	26	7	33	15
<i>Thermi</i> ; c – <i>Deinococci</i> ; o – <i>Thermales</i> ; f – <i>Thermaceae</i> ; g – <i>Thermus</i>	52	7	59	20

Single partner interactions denote how many phages are exclusively interacting with that particular bacterium.

control, and similar avenues of investigation may potentially prove fruitful in controlling disease outbreaks in coral culturing facilities. Phage–bacterial networks can be used to predict which phages may be useful against pathogenic bacteria, while also preventing loss of beneficial mutualist bacteria. Questions about the host range of bacteriophage have been asked for some time (Frisch and Levine, 1936) and phages have been shown to have both narrow and broad host range (Yarmolinsky and Sternberg, 1998; Jensen *et al.*, 1998; Holmfeldt *et al.*, 2007; Flores *et al.*, 2011; Weitz and Wilhelm, 2012; Flores *et al.*, 2012) while similarly, bacteria are commonly susceptible to multiple phage strains, which can be evolutionary and/or geographical distant (Flores *et al.*, 2012).

Network analysis can be used to navigate these complex interactions, for example by detecting phages that are predicted to only affect bacteria of interest. For example, *Rhodobacterales* and *Campylobacteriales*, the two bacterial orders associated with WP in this study, had six phages that were not only shared in common, but had the same interaction type; thus, they would make potential candidates for phage therapy (Fig. 4, Fig. S5).

In the case of the disease coral-associated *Rhodobacterales* and the healthy coral-associated *Kiloniellales*, it is interesting that seven phages were shared between them (Fig. 4, Fig. S6). However, six of the seven phages that were interacting with both bacteria had different interaction types. Therefore, it is possible that the presence of phages is either preventing the proliferation of one of those bacterial types in a healthy coral state (i.e. controlling *Rhodobacterales* populations), or the absence of phages is allowing uninhibited growth of these bacteria in a disease state (i.e. no control of *Rhodobacterales* populations). Interestingly, Barr and colleagues (2013) demonstrated that coral mucus con-

tains receptors for bacteriophages, positing that mucus may retain beneficial phages. Perhaps a disease state interrupts the production of these receptors or alters the mucus such that the phages are not attaching, leading to disproportionate growth of detrimental bacterial types. The addition of beneficial phages may have potential to reduce disease-associated bacteria in cases where the natural defense mechanisms fail.

#### *Methodological considerations for phage–bacterial network analysis*

This work is an example of the utility of comparing 16S rRNA amplicons to physically purified viral metagenomes for network analysis. Use of these distinct pools avoids many issues that would arise if characterizing phage and microbes from shotgun metagenomic data alone. If a single pool of metagenomic data for each sample (containing a mixture of phage and bacterial DNA) was used to infer correlations, many observed sequences with similarity to phage genes could in fact reside in bacterial chromosomes. This could induce false-positive correlations between phage and bacterial taxa that happen to carry phage genes. In this study, purification of viral and phage particles by density gradient centrifugation, and separate analysis of bacterial/archaeal diversity with 16S rRNA gene amplicons, greatly reduced the opportunity for such false-positive correlations. Yet it is still possible that some low level free microbial DNA (e.g. from lysed cells) could have made its way into the preparation. Physical separation of viral particles by CsCl gradient centrifugation may still include gene transfer agents (GTAs) produced by some bacteria (especially *α-Proteobacteria*). Yet because these phage-like particles package only random regions of DNA rather than preferentially packaging phage DNA

(reviewed in Zaneveld *et al.*, 2008; Lang *et al.*, 2012), most genes carried by these GTAs will be bacterial in origin. Therefore, any DNA sequences carried by GTAs would have been filtered out during similarity searches against viral genome databases, except in the relatively rare cases where the GTA happens to package a phage gene (e.g. a lysogenic phage resident in the GTA-producing bacteria's genome), or a bacterial gene that happens to also be carried by a reference viral genome. To control for this, we both screened the purified viral particles for bacterial contamination using 16S rRNA gene primers, and examined sequenced phage genes for known bacterial genes using DeconSeq.

Following separation of viral particles and DNA extraction, multiple displacement amplification (MDA) was used to obtain sufficient DNA for sequencing. As with many amplification techniques [notably including polymerase chain reaction (PCR), e.g. Bergmann *et al.*, 2011], MDA may produce a biased estimate of true relative abundances. However, the most comprehensive studies of MDA bias, using mock communities where true community composition is known, suggest that this bias (while real) is reproducible across samples (mock community  $R^2 \geq 0.98$  between technical replicates, Yilmaz *et al.*, 2010;  $R^2 \geq 0.97$  between MDA replicates, Marine *et al.*, 2014). Since our study compared estimated relative abundance patterns across samples, reproducible MDA biases would affect all samples, and therefore not change patterns between samples. For the network construction, we used correlations in estimated relative abundances across samples. Consistent amplification of abundance of any given phage in all samples should not therefore cause spurious correlations. For example in a linear (Pearson) regression, such amplification should affect the intercept but not the slope or  $R^2$  of the correlation between phage and bacteria. Thus, our interpretation is that this technique is appropriate for the questions addressed here.

#### *Phage taxonomic and database considerations for inferences in phage–bacteria interactions*

One additional consideration in this network analysis is that many environmental phages are quite divergent from characterized phages (Rohwer and Edwards, 2002; Rohwer *et al.*, 2002; Tucker *et al.*, 2010). Given the vast viral and phage diversity found in nature, the range of possible phage sequences likely vastly exceeds what is currently available in databases. Therefore, it is likely that some phage found in corals do not have any representation in sequence databases. These phage will not be captured by our fairly conservative (for viral/phage studies) BLAST e-value threshold of  $1e-5$ . Such cryptic diversity can be substantial in underexplored systems. For example, 78.85% of reads were unassigned in a

recent study using the same methods on coprolites, which also used an e value  $1e-5$  BLAST threshold (Appelt *et al.*, 2014). Here ~21% of reads showed high similarity to known groups.

Although this is a current limitation of the field, methods of circumventing this issue using unsupervised clustering and/or k-mer analysis show promise for future development. Yet a current limitation of such unsupervised (non-reference-based) approaches, particularly in host–symbiont systems, is that any contamination such as non-phage viruses or residual free DNA would then potentially be incorporated into the analysis as separate clusters, whereas such sequences would be excluded by the reference-based approach used here. Because we focus on phage specifically, and because our study system exists in intimate association with both the coral host tissue and *Symbiodinium*, we pursued the more conservative approach of studying only those gene sequences with similarity to known phage genes. Despite the conservative nature of this approach, we were still able to detect interesting and potentially relevant co-occurrence patterns with bacterial taxa.

Taxonomic annotation for reference sequences also bears careful consideration. Of the phage genes that we sequenced, we can only know for sure that they share sequence similarity with known phages. Phage strains are named for host of isolation, but the same phage strain can often infect multiple hosts (e.g. Weitz *et al.*, 2013). Therefore, we distinguish sequence similarity to a phage isolated from a particular bacterium from the true host(s) for a given environmental phage. Therefore, we did not rely on annotated names for interpreting which specific phages are interacting with bacteria, instead focusing on co-occurrence patterns of sequences in the data. Phage names such as 'Clostridium-phage-phiSM101' are provided for information, but do not necessarily indicate that a particular environmental phage infects that host to the exclusion of others. Thus, one advantage of co-occurrence network analysis is that these difficulties with phage taxonomy assignment are largely side-stepped: inferences are drawn from correlations in the sequence data, rather than taxonomy.

To address the issue of direct versus indirect interactions, we attempted to create bacteria–bacteria networks to extrapolate whether phage interactions were indirect or direct. However, no interactions were found significant for just the 11 bacterial genera of interest, all bacterial genera and the combination of those two across all samples using the same conservative methods as the phage–bacteria network, or even with relaxing the FDR correction up to 0.2. This suggests that the predicted phage–bacteria interactions are likely direct (given lack of interactions among bacteria), or that sample sizes were too small without the phage data.

### Phage–bacterial networks as a tool for studying the microbial ecology of diseased organisms

Our presented approach to evaluate patterns among individuals of various health states to infer interactions among the phage and bacteria could be complemented by longitudinal studies (e.g. Chow *et al.*, 2013) that look at patterns of change across time. Such an ideal experiment of monitoring microbial/phage dynamics in host disease models would require predicting which individuals/areas will become infected at given times, thereby capturing disease dynamics in real time. This would require intermittent sampling and monitoring of healthy individuals before infection, followed by intensive sampling when disease outbreaks are first detected. Infection experiments in a controlled environment may be an alternative approach to evaluate phage–bacteria dynamics over the course of a given disease.

This study is an initial endeavor that can serve as a catalyst to experimental tests of these proposed interactions (when these phages are successfully cultured) and more in-depth network studies of coral phage–bacteria interactions over time. Despite these caveats, networks remain a valuable and useful tool to answer questions about phage–bacteria interactions that currently cannot be answered in any other way with limited corals for sampling, and lack of cultured coral bacteria/phages.

### Conclusions

The aims of this study were twofold, first to describe the bacterial and bacteriophage consortia across different coral health states, and second to infer interactions among the phages and bacteria. This study determined that both bleached coral tissues versus diseased tissues had distinct microbial communities. Diseased tissues harboured an increased relative abundance of the genus *Arcobacter* in the order *Campylobacterales* and an unknown genus in the order *Rhodobacterales* compared with the other health states. Diseased corals also showed decreases in the abundance of one unknown genus in the order *Kiloniellales*. The bacteriophage consortia at the strain level demonstrated similar clustering patterns as the bacterial (genus level) communities. In addition, phages with similarities to *Inoviridae* were elevated in healthy and diseased tissues compared with bleached. Using phage–bacteriophage interaction networks, we were able to predict which phages were interacting with the bacteria genera shown to vary across health states, and therefore potentially controlling their abundances. Ultimately, this study has taken a multifaceted approach to describe and explain bacterial community changes post-stressors in corals, by looking at phages and bacterial communities concurrently. Finally, this study also highlights the potential for phage–bacterial networks to aid in

selection of therapeutic phages that target opportunistic pathogens without negatively influencing beneficial bacteria through interactions.

## Experimental procedures

### Sample collections

Coral samples were collected using SCUBA, where two to three cores of tissue attached to skeleton were removed from each *M. annularis* colony (Fig. 1) using a 2 cm diameter corer and hammer (USVI Department of Planning and Natural Resources permit #STT-050-10). Samples were collected at depths of 5.5–7.6 m and at temperatures ~29°C. Colonies were within 5–7 m of each other and ~75 m from shore. Disease in this paper refers to the appearance of WP signs, while coral bleaching is defined as the pale or white appearance of colonies typically due to the loss of *Symbiodinium* and/or chlorophyll from coral tissues (Brown, 1997). In Soffer and colleagues (2014), WP infections were defined as: (i) lesions consisted of an area of recent tissue loss where denuded skeleton with little to no algal colonization (that could indicate past and unrelated tissue loss) was delineated from living tissue by a smooth, undulating margin, (ii) lesions were located peripherally or basally on the colony, and (iii) lesions expanded at a mean rate (0.23 cm/day ± 0.12) similar to those in reports of WP type I disease (Dustan, 1977; Sutherland *et al.*, 2004). These disease signs and epidemiological properties are consistent with those previously reported for WP (reviewed in Sutherland *et al.*, 2004; Bythell *et al.*, 2004; Brandt *et al.*, 2013). Disease was monitored before and after collection. The expanding zone of the lesions confirmed active infection and the likely presence of the culprit pathogen(s). Because samples were collected during an active bleaching event, all colonies that showed signs of WP were also bleached on the top of the colony. However, our sampling distinguishes tissues at the boundary of WP lesions or affected by bleaching. From the seven WP diseased colonies, tissues were cored from both the bleached top 'bleached + diseased (BD)' and the margin of lesions 'diseased (D)', which did not show signs of bleaching. Tissue from an additional six 'bleached (B)' non-WP diseased corals and two 'healthy (H)' corals (normal pigmentation, non-diseased) were taken as controls (Fig. 1). Coral plugs were rinsed with 0.02 µm filtered seawater. For virome libraries, tissue was removed by airbrushing with ~40 ml of 0.02 µm filtered phosphate buffer saline solution (pH 7.3). Tissue homogenates were 0.22 µm filtered, preserved in molecular biology grade chloroform (2% final concentration), and stored at 4°C until viral metagenome generation processing. For bacterial libraries, an intact coral plug was rinsed with 0.02 µm filtered seawater and preserved in 95% ethanol until extractions.

### Bacterial libraries

Most of the tissue sloughed off during ethanol preservation; therefore, 500 µl of ethanol/tissue slurry was pipetted for DNA extractions. A modified organic extraction protocol that excluded the tRNA and phenol steps was used to purify DNA

(Rowan and Powers, 1991). Isolated nucleic acids were amplified using barcoded primers 515F and 806R, which were chosen due to their ability to amplify both bacteria and archaea (Caporaso *et al.*, 2011; Walters *et al.*, 2011). Triplicate amplicon libraries were prepared using GoTaq Flexi reagents from Promega (Madison, WI, USA) using standard protocols and the following PCR cycle: 1 cycle of 94°C for 3 min, 35 cycles of 94°C for 45 s, 50°C for 60 s and 72 °C for 90 s, and then 1 cycle of 72°C for 10 min. PCR products were run on a 1.5 agarose gel, triplicates pooled, cleaned using AMPure magnetic beads from Agencourt (Brea, CA, USA) and quantified with a Qubit HS dsDNA kit (Invitrogen, Eugene, OR, USA) into equimolar ratios. Quality of amplicons were determined on an Agilent Bioanalyser 2100 before being pyrosequenced on a 454 GS Junior Roche at the Oregon State University Center for Genome Research and Biocomputing Core Laboratories.

### 16S rRNA gene analysis

16S rRNA gene sequence libraries were analysed in QIIME and PRIMER 6 (Clarke and Gorley, 2006; Caporaso *et al.*, 2010). First, reads were de-multiplexed based on their error-correcting barcodes and filtered for quality using default parameters (quality score  $\geq 25$ , min length = 200, max length = 1000, and no ambiguous bases and mismatches allowed). Next OTUs were assigned at a 97% similarity threshold UCLUST (Edgar, 2010), and OTU tables constructed from the assignments. Lastly, taxonomic annotations were made to the RDP database using the RDP classifier (Wang *et al.*, 2007). OTUs identified as chloroplasts were removed from the analysis (mean  $23\% \pm 0.2$ ). Amplicon libraries were paired with all virome samples with the exception that there was an additional B sample processed for bacterial analysis.

### Viral metagenome generation

Phage particles were isolated and sequenced separately from bacterial cells (using CsCl density gradient ultracentrifugation) before sequencing (Vega Thurber *et al.*, 2009; more details in Soffer *et al.*, 2014). DNA was then extracted with an phenol-chloroform extraction protocol (Vega Thurber *et al.*, 2009; Soffer *et al.*, 2014) and amplified using non-specific MDA according to manufacturer's protocol (GenomPhi, GE Healthcare, Pittsburgh, PA, USA). PCR reactions using 16S and 18S primer sets were performed to determine whether bacterial or eukaryotic DNA contaminated the viromes. No contamination was detected.

The coral phage libraries (21 coral samples) were barcoded and pyrosequenced at EnGencore (University of South Carolina) on a Roche Titanium 454 platform. The final numbers of replicate libraries for each coral health state were: H ( $n = 2$ ), B ( $n = 5$ ), BD ( $n = 7$ ) and D ( $n = 7$ ).

### Phage library processing and bioinformatic analyses

Sequence reads underwent several preliminary bioinformatic steps. SFF files were converted to FASTA/FASTQ files and de-replicated using the program GALAXY (Goecks *et al.*,

2010). Low-quality reads (i.e. those  $< 100$  base pairs in length and/or with quality scores  $< Q20$ ) were removed. Using the CAMERA (Community Cyberinfrastructure for Advanced Microbial Ecology Research and Analysis) (Sun *et al.*, 2011) platform, the tBLASTx algorithm was used to find similarities to sequences in the National Center for Biotechnology Information non-redundant viral database (Altschul *et al.*, 1990). This annotation level will be referred to as 'strain level'. Family level taxonomic was manually assigned for the strongest similarity (best tBLASTx e-value  $\leq 10^{-5}$ ) identified to a known viral genome. For this study, only sequences similar to bacteriophage were utilized for network analysis (for eukaryotic viral analysis, see Soffer *et al.*, 2014).

### Community and consortia statistical analyses

A summary table of relative bacterial abundance (genus level) or bacteriophage relative abundance (strain level) were used to analyse microbial community and viral consortia structure.  $\beta$ -Diversity of normalized and log-transformed Bray–Curtis distances were summarized in non-metric multi-dimensional scaling plots (25 iterations). An ANOSIM was used to test for significant differences among overall bacterial or bacteriophage communities (Clarke and Warwick, 2001). Similarity percentage (SIMPER) (Clarke and Warwick, 2001) analysis using Bray–Curtis distances was used to determine which bacteria or phage taxa contributed to dissimilarity. All of these were computed in Primer 6 (Clarke and Gorley, 2006). The phage or bacterial taxa that drove differences between these clusters were identified with SIMPER analyses. Significant differences were subsequently tested using Kruskal–Wallis and a Bonferroni *post hoc* test (if  $P \leq 0.05$ ) in Analyse-It (V2.3).

### Construction, visualization and analysis of phage–bacteria interaction networks

Significant correlations between phage strains and bacterial genera were identified using the CoNet Cytoscape plug-in (Shannon *et al.*, 2003; Faust and Raes, 2012; Faust *et al.*, 2012). Co-occurrence or mutual exclusion interactions were identified using an ensemble of correlation measures of a recently described bootstrap and renormalization approach to reduce false positive and compositionality biases (Faust and Raes, 2012; Faust *et al.*, 2012). In this study, available sample sizes did not provide sufficient statistical power to allow inference of separate networks for each health state individually ( $q > 0.05$  for all health state networks). In order to focus on bacteria potentially associated with disease and/or bleaching, bacterial genera of interest for the network analysis were chosen based on whether they were significantly different in any one of the health states; this was determined using ANOVA and a FDR of 0.2 in QIIME (Benjamini and Hochberg, 1995). For all phage–bacteria pairs, correlation or dissimilarity scores were calculated using Pearson and Spearman correlation, or Bray–Curtis and Kullback–Leibler dissimilarities respectively. Annotated bacteriophage strain level relative abundance and bacterial 16S genus abundance (normalized to column sum) were input as separate matrices for all health states combined, with a minimum row filter (for zeros) of 3. The ReBoot

procedure using 100 permutations was used to control for potential false-positive correlations due to the compositional nature of relative abundance data (Faust and Raes, 2012; Faust *et al.*, 2012). The resulting distribution was run with 1000 bootstraps. Finally, an FDR correction (Benjamin Hochberg,  $P$ -value threshold or  $q \leq 0.05$ ) was applied to the  $P$ -value of all correlations based on the number of phage strain/bacterial genus pairs tested to control the FDR for multiple comparisons. In each analysis, the  $P$ -value for correlations was combined across multiple correlation measures (Pearson, Spearman, Bray–Curtis, Kullback–Leibler) using Simes method (Sarkar and Chang, 1997), and only correlations supported by at least two correlation methods were included. Variances were pooled.

Inferred interactions between phage and bacteria were visualized as a network in Cytoscape (Shannon *et al.*, 2003). Networks were arranged using the Edge-weighted spring algorithm, which treats each node as connected by springs of varying strength (bacteria and phages with more interactions are closer together). The Cytoscape plug-in NetworkAnalyzer (Assenov *et al.*, 2008) was used to compute the following: node degree distribution (power law fit), average shortest path length (character path) and heterogeneity. Degree node distributions were fit to the power law using NetworkAnalyzer to determine whether networks exhibited scale-free properties; networks exhibiting scale-free properties increase in size exponentially with increasing nodes, and nodes are more likely to attach to already existing nodes (Barabási, 1999). The correlation value ( $R^2$ ) was calculated based on logarithmic values. Modularity was not tested, as we did not infer interactions among the phages, or interactions among the bacteria, and modularity is computed by these same microbe-type interactions (Ravasz *et al.*, 2002).

Comparisons among individual health state networks did not yield supported and consistent networks, due to low sample size, and therefore are not presented. We also tested whether phage families overall show co-occurrence patterns with bacterial families or OTUs. No significant correlations were found between phage families and bacterial families or bacterial OTUs, likely suggesting that phage host range (or other factors affecting co-occurrence patterns) are not conserved across phage families. This is consistent with the observation that members of each phage family can infect phylogenetically diverse bacterial hosts. We therefore focus on the more specific phage strain-OTU data.

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### Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Fig. S1.** Bleached and diseases bacteriophage consortia differ. Non-metric multidimensional scaling (MDS) plot of bacteriophage consortia from different health states at the phage strain level. Log-transformed Bray–Curtis similarity resemblance matrix was used to construct the MDS (2D had high stress value therefore 3D is reported here). Bacteriophage consortia showed similar overall clustering patterns to bacterial genera (Fig. 2): D tissues cluster significantly different from B and BD (ANOSIM  $P \leq 0.05$ : Global R = 0.233), but no tissue type clustered significantly separately from H (ANOSIM  $P \leq 0.05$ : Global R = 0.233). Reported stress value is based on 3D MDS.

**Fig. S2.** Mean relative percent of bacteriophage families (and unclassified). Letters denote significantly different relative percent abundances out of 100% (Kruskal–Wallis,  $P \leq 0.05$ ). N.S. are not significantly different. Error bars are standard error of the mean. Colours of the bars indicate: healthy (red); bleached (yellow); bleach + diseased (green); diseased (blue).

**Fig. S3.** Node degree distribution fitted line based on power law demonstrates scale-free properties. The fitted line is based on logarithmic scale and was calculated the NetworkAnalyzer plug-in in the format of  $y = ax^b$ , where  $a = 38.526$ , and  $b = -1.098$ .

**Fig. S4.** Phage specificity based on predicted network. Each bar represents the number of bacterial hosts that a phage was predicted to interact with. Most phages only interacted with one bacterial host. Co-occurrence bars are green, and mutual exclusion bars are red.

**Fig. S5.** Phage–bacteria interactions of two bacteria associated with diseased tissues. Edges and nodes isolated from the original network (Fig. S4). Green lines represent co-occurrence interactions, while red lines represent mutual exclusion interactions.

**Fig. S6.** Phage–bacteria interactions of *Kiloniellales* (yellow square) and *Rhodobacterales* (orange square) only. Edges and nodes isolated from the original network (Fig. S4). Green lines represent co-occurrence interactions, while red lines represent mutual exclusion interactions.

**Table S1.** Bacterial and Archaeal phyla present in different coral health states. 16S rRNA gene amplicon sequences were clustered into OTUs at 97% sequence identity, and the representative sequence for each OTU was assigned taxonomy using the RDP classifier at a 70% confidence threshold.

**Table S2.** Number of bacteria with which each phage interacts. Interactions are supported by at least two correlation metrics (Pearson, Spearman, Bray–Curtis or Kullback–Leibler) and  $P \leq 0.05$ . Phage annotations best on tBLASTx best hit (e-value  $\leq 10^{-5}$ ).